Supporting Information

Elrouby and Coupland 10.1073/pnas.1005452107

SI Materials and Methods

Yeast Two-Hybrid Screens. PCR was used to create the ESD4 truncations. Primers ESD4-1S (5′-GGGCCATGGGTGCCGATGCG-3′), ESD4-2A (5′-GACCCGGGTCAGTTTTCATGAGTAGCC-3′), ESD4-3S (5′-GGGCCATGGAAAACTCAAACATTG-3′), and ESD4-4A (5′-GACCCGGGTCAATCAGCTCGTAGC-3′) were used to amplify sequences encoding the N-terminal 285 amino acids and the C-terminal 207 amino acids, respectively. Sense and antisense primers contained an Nco1 site, an engineered stop codon, and a Sma1 site. The SCE or ESD4 coding region was cloned into the Nco1-Sma1 sites of the yeast two-hybrid vector pGBKT7 and sequenced to confirm in-frame fusions with the GAL4 DNA-binding domain. Bait plasmids were introduced into the yeast strain PJ694α and mated with PJ694a containing two *Arabidopsis* cDNA libraries fused to the GAL4 activation domain in pGAD-T7 (generated using oligo-dT or random primers; a gift from Hans Sommer, Max Planck Institute).

***E. coli* SUMOylation Assays.** Full-length cDNA clones encoding potential SUMO substrates were obtained from the RIKEN BioResource Center (1). cDNAs were further amplified using primers containing the gateway (GW) cassettes (Invitrogen) and cloned into pDONR201. The Duet cloning system (Novagen) was used to reconstitute the *Arabidopsis* SUMO pathway in *E. coli*. Genes encoding the two subunits of SAE (SAE1b and SAE2) were cloned into the Nde1-Xho1 and the Sac1-Pst1 sites of multiple cloning sites (MCS) 2 and 1 of pCDF, respectively, to generate pCDF-SAE. SCE and either SUMO1 or SUMO3 were cloned into the Nco1-HindIII and Nde1-Xho1 sites of pACYC MCS1 and 2, respectively, to generate pACYC-SCE-SUMO. Putative substrates were cloned into pET32b-GW as fusions with N-terminal Trx and 6-HIS tags (Novagen). As controls, we used site-directed mutagenesis to convert the active site cysteine of SCE into serine (C94S) and used a pCDF vector, into which only one of the two SAE subunits was cloned (SAE1b). *E. coli* BL21 (DE star) cells (Novagen) were transformed with both pCDF-SAE and pACYC-SCE-SUMO or pACYC-SCE(C94S)-SUMO. pET32b-GW containing the test substrate was then introduced into these strains. To induce expression of all genes and SUMOylation of the substrate, cultures were grown at 37 °C for 2 h, induced with 1 mM IPTG, and incubated at 28 °C overnight and then at 25 °C for 2 h. For detection by Western blot analysis, substrate proteins were purified on nickel nitriloacetate resin in 8 M urea (Qiagen). Proteins were separated on 10% SDS-PAGE and blotted onto PVDF membranes (Millipore) using standard methods. Blots were blocked for 2 h in PBS with 5% nonfat milk, after which anti-Trx (Sigma-Aldrich) or anti-SUMO antisera (2) were added. After 2 h, the blots were washed twice in PBS-milk, and a secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase; Sigma-Aldrich) was added. After washing, the blots were treated with BCIP and NBT to visualize the signals.

In Vivo SUMOylation of ADA2b. Heterozygote lines containing a null T-DNA allele of *ADA2b* (*ada2b-1*) and a full-length ADA2b cDNA were gifts from Steven Triezenberg (Michigan State University) and Christian Luschnig (University of Vienna), respectively. The cDNA was amplified using primers containing the GW cassette fused to ADA2b-S (5′ATGGAGGCCGAATTCCCG-3′) or ADA2b-A (5′-TTAAAGTTGAGCAATACC-3′) and cloned into pDON201. The cDNA was transferred into the destination binary vector pAlligator-2 (2) generating an N-terminal fusion with 3× HA tag driven by CaMV 35S promoter. pAlligator-ADA2b was introduced into *Agrobacterium tumefaciens* strain GV3101, and the resulting cells were used to transform the *ada2b-1* heterozygote line (3). T1 plants segregating for the *ada2b-1* T-DNA were screened as described previously (4), and homozygous lines that also expressed the GFP seed coat marker of pAlligator were selected. Transgenic plants expressing CDF2 as an N-terminal fusion with 3× HA and under the control of the sucrose transporter 2 (SUC2) promoter were a gift from Fabio Fornara (Max Planck Institute).

For Western blot analysis and immunoprecipitation, 12-d-old seedlings were grown on MS agar under controlled conditions (22 °C, 12 h light/12 h dark). Seedlings were ground in liquid nitrogen and two volumes of extraction buffer [20 mM Tris (pH 6.8), 5 mM $MgCl_2$, 50 mM NaCl, 150 mM KCl, 1 mM EGTA, 0.5% Triton X-100, 0.02% Nonidet P-40, 1 mM PMSF) and 1:200 plant protease inhibitor cocktail (Sigma-Aldrich) were added. After a brief centrifugation step to pellet tissue debris, the total protein extract was separated on 10% PAGE and blotted onto PVDF membranes. Immunoprecipitation using the Sigma-Aldrich anti-HA IP kit was followed by Western blot analyses using the anti-HA antibody (Sigma-Aldrich) or the anti-SUMO1 antibody (2), and the signal was detected using the Pierce Femto substrate system.

Database and Gene Ontology Searches. We used a simple Perl line to search the *Arabidopsis* proteome (release 7) for proteins containing the sequence I/V/LKxD/E. This sequence defines an hpSAS (≥91%) (5). To characterize the putative SUMO substrates, we used both the BLAST algorithm and Gene Ontology searches of the *Arabidopsis* Information Resource. To test for potential enrichment of classes of genes involved in specific biological processes, we used FatiGO of the Babelomics suite of programs (Babelomics 3.2) (6).

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3. Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743.
4. Vlachonasios KE, Thomashow MF, Triezenberg SJ (2003) Disruption mutations of *ADA2b* and *GCN5* transcriptional adaptor genes dramatically affect *Arabidopsis* growth, development, and gene expression. *Plant Cell* 15:626–638.
5. Xue Y, Zhou F, Fu C, Xu Y, Yao X (2006) SUMOsp: A Web server for sumoylation site prediction. *Nucleic Acids Res* 34(Web server issue):W254–W257.
6. Al-Shahrour F, et al. (2007) FatiGO+: A functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. *Nucleic Acids Res* 35(Web server issue):W91–W96.

Other Supporting Information Files

Table S1 (XLS)
Table S2 (XLS)